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ACTIVE MOTIF®

Tools to Analyze
Nuclear Function

Magnetic Beads Make Chromatin Immunoprecipitation Faster and Easier

Active Motif's ChIP-IT™ Express Kits use protein G-coated magnetic beads instead of the traditionally used agarose beads to make chromatin immunoprecipitation (ChIP) simpler and faster than ever before. The use of magnetic beads has made it possible to simplify and streamline the ChIP protocol, eliminate a number of time- and labor-intensive steps and dramatically reduce background. The result is that ChIP-IT Express makes your ChIP experiments more successful and enables you to use less starting material.

ChIP method

ChIP is a powerful tool for studying protein/DNA interactions because it combines the specificity of immunoprecipitation, the sensitivity of PCR and the power of array profiling. In ChIP, intact cells are fixed using formaldehyde, which cross-links and therefore preserves protein/DNA interactions at that precise time point. The DNA is then sheared into small fragments using either sonication or enzymatic digestion and specific protein/DNA complexes are immunoprecipitated using an antibody directed against the DNA-binding protein of interest. Following immunoprecipitation, the chromatin is eluted, cross-linking is reversed and the proteins are removed by treatment with Proteinase K. The DNA is then analyzed, most commonly by PCR, to determine which DNA fragments were bound by the protein of interest (Figure 1).

While it is a powerful technique, performing traditional ChIP is a complicated, multi-day procedure that is not well suited for the analysis of numerous samples. Moreover, troubleshooting the procedure and interpreting ChIP results can be difficult without validated reagents and controls that have been proven to work in ChIP.

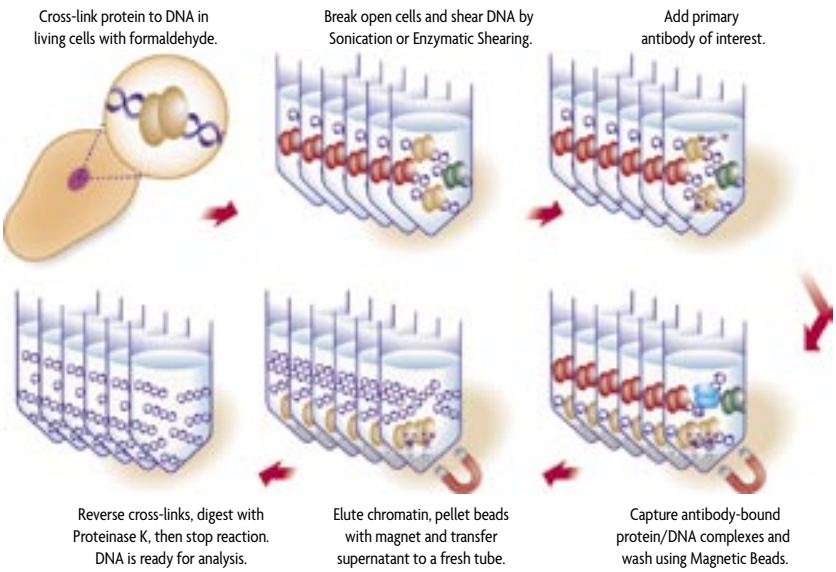


Figure 1: Flow chart of chromatin immunoprecipitation with ChIP-IT Express.

ChIP-IT Express improves traditional ChIP

All standard ChIP methods utilize agarose-based affinity beads to capture the antibody/chromatin complexes. However, spin-washing these beads is slow and tedious. In addition, DNA isolated through these methods must be further purified (by spin columns or phenol/chloroform extraction & precipitation) prior to PCR analysis.

We have developed a greatly simplified ChIP protocol that utilizes magnetic protein G-coated beads for antibody capture. Magnetic beads enable a streamlined ChIP protocol that has fewer steps, requires little hands-on time and is compatible with multi-channel pipetting, which makes high-throughput ChIP a reality (Table 1).

Table 1: Comparison of Standard ChIP vs. ChIP-IT Express protocols.

Standard ChIP protocol	ChIP-IT Express protocol
Prepare chromatin by sonication	Prepare chromatin by sonication or enzymatic digestion
Block protein G beads, then use beads to pre-clear chromatin	
Combine chromatin and antibody; incubate 4 hours or overnight	Combine chromatin, antibody and magnetic beads; incubate 4 hours
Add blocked protein G beads and incubate 1.5 hours	
Wash beads 8 times (each wash: pellet beads with a 2-minute centrifugation, let beads settle, remove supernatant, resuspend)	Wash beads 3 times (each wash: pellet magnetic beads in seconds with magnet, remove supernatant, resuspend in wash buffer)
Elute chromatin from beads using two 15-minute elutions	Elute chromatin (15 minutes), pellet beads in seconds with magnet and transfer supernatant to a fresh tube
Reverse cross links (4 hours at 65°C)	Reverse cross links (15 minutes at 94°C)
Add Proteinase K to digest proteins (1.5 hours at 42°C)	Add Proteinase K to digest proteins (1 hour at 37°C)
Purify DNA by 2 phenol/chloroform extractions, followed by precipitation and resuspension	Add Proteinase K Stop Buffer
DNA is ready for analysis (*e.g.* PCR)	DNA is ready for analysis (*e.g.* PCR)

Step 1: Cell fixation

The first step in ChIP is fixing the cells that are to be studied through the use of formaldehyde; this cross-links the DNA-binding proteins to the DNA so that the protein/DNA complexes can be immunoprecipitated. While this is a fairly straightforward step, over-fixation is a common problem. When this occurs, the chromatin becomes resistant to shearing, which prevents efficient shearing of the chromatin in the next step of the protocol. The ChIP-IT Express Kit includes all buffers (excluding formaldehyde) required for cell fixation. A Glycine "Stop-Fix" Buffer is included to prevent excessive cross-linking and Protease Inhibitor Cocktail (PIC) and PMSF are included to ensure that the protein/DNA interactions are preserved throughout the procedure.

Step 2: Chromatin shearing by sonication or enzymatic digestion

After fixation, the chromatin is sheared into small fragments. This has traditionally been performed by subjecting the chromatin to different pulses of sonication. Although sonication is an effective method for shearing DNA, it can be difficult to optimize due to complications arising from emulsification and overheating. And, because the quality of your sheared sample depends greatly upon the quality of your sonicator, it may be necessary to purchase an expensive, "high-end" sonicator to get reproducible shearing. Because of this, Active Motif has developed an enzymatic method for shearing chromatin, which we believe is more reproducible and user-friendly. Our proprietary Enzymatic Shearing Cocktail quickly shears DNA into 200-1000 bp fragments (Figure 2). Because enzymatic shearing is solely time and temperature dependent, it is more consistent and the problems caused by sonication are eliminated, so ChIP results are improved.

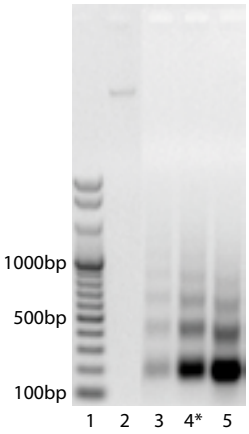


Figure 2: Analysis of DNA sheared using the Enzymatic Shearing Kit.
HeLa cells were fixed for 10 minutes with 1% formaldehyde and then chromatin was prepared using the ChIP-IT Express Enzymatic Kit protocol. Chromatin was sheared with the Enzymatic Shearing Cocktail for 5, 10 & 15 minutes and the reaction was stopped with the addition of cold EDTA. The sheared and unsheared chromatin samples were subjected to cross-link reversal, treated with Proteinase K, phenol/chloroform extracted and precipitated as described in the protocol. Samples were separated by electrophoresis through a 1% agarose gel.

Lane 1: 100 to 1000 bp ladder.
Lane 2: Unsheared HeLa DNA.
Lane 3: HeLa DNA treated for 5 minutes (under-digested).
Lane 4: HeLa DNA treated for 10 minutes (optimized digestion).
Lane 5: HeLa DNA treated for 15 minutes (over-digested).
***Note**: From this experiment, the DNA treated for 10 minutes was optimal and used successfully in ChIP.

ChIP-IT Express Kits are offered with your choice of either sonication or enzymatic shearing reagents. Because chromatin from different cells lines behaves differently, the kits provide reagents sufficient for performing 3 optimizations, then making 5 preparations of sheared chromatin. Each preparation of sheared chromatin requires three 15 cm tissue culture plates of cells and yields chromatin sufficient for at least 16 ChIP-IT Express reactions (one ChIP reaction is considered to be the incubation of

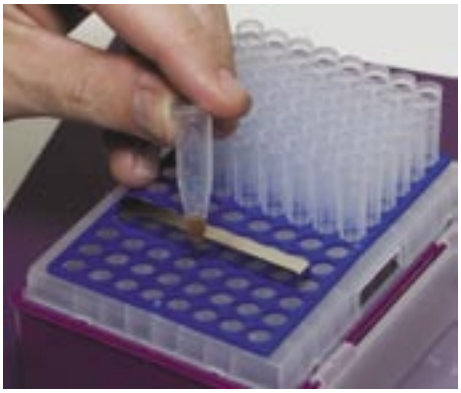


Figure 3: Use of a standard tip box for magnetic ChIP.
ChIP-IT Express Kits include a strong bar magnet that can be used to convert tip boxes into a magnetic stand for washing the protein G-coated magnetic beads.

one sample of chromatin with one antibody). If you wish to prepare chromatin from a smaller number of plates, simply scale the protocols down accordingly.

Step 3: Chromatin capture on magnetic beads

In traditional ChIP, the next steps would be to block the protein G agarose beads and then to incubate the blocked beads with the sheared sample. This pre-clearing step is designed to reduce the non-specific background binding of proteins to the protein G and beads.

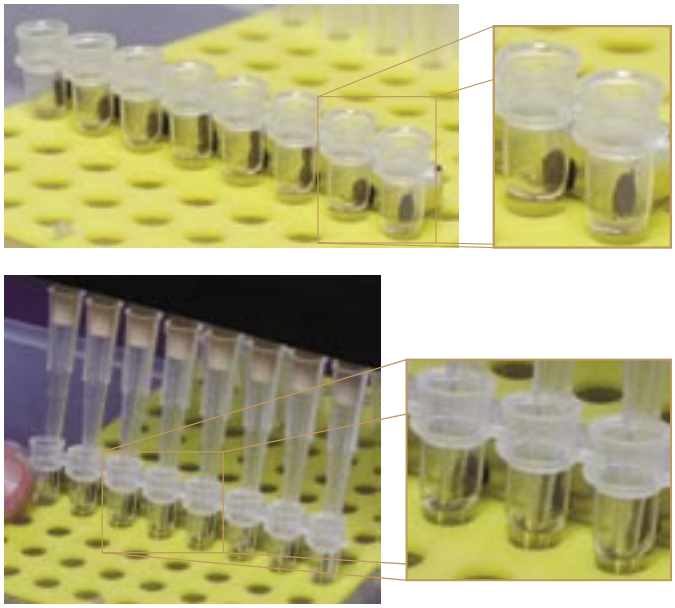
In contrast, the protein G-coated magnetic beads in ChIP-IT Express Kits are supplied ready-to-use, so there is no need for blocking. As the magnetic beads have a very high binding capacity for IgG, they are used in smaller volumes that reduce non-specific binding. Because of this lower non-specific binding, it is not necessary to pre-clear the chromatin. With ChIP-IT Express, you can skip these two steps and proceed immediately to antibody capture. The beads are simply added to the sheared chromatin along with the antibody against the DNA-binding protein of interest. And, incubation time is only 4 hours, as opposed to overnight for traditional ChIP assays.

Steps 4 & 5: Chromatin immunoprecipitation and washing

After incubation of the antibody, beads and chromatin, the protein/DNA complexes are immunoprecipitated and washed. This is extremely simple with ChIP-IT Express because the magnetic beads pellet in seconds simply by placing the tube next to the magnet that is provided in the kit (Figure 3). An added advantage is that the magnetic beads pellet onto the side of the tube. This makes it easy to remove buffers without disturbing the beads.

After immunoprecipitation and removal of the supernatant, the chromatin and beads are washed. ChIP-IT Express saves a lot of time and effort because magnetic beads require fewer washing steps than agarose beads (3 washes vs. 8). Moreover, while agarose beads must be pelleted by a 2-minute centrifugation, the magnetic beads pellet in seconds. This dramatically reduces hands-on time and ensures sample-to-sample consistency. The provided siliconized microcentrifuge tubes (1.7 ml) ensure a minimal loss of beads during the procedure.

The included magnet can be used to turn pipette tip boxes into magnetic stands; P1000 tip boxes are used with Eppendorf tubes, while P200 boxes can be used with 8-well PCR strips to process multiple samples using a multi-channel pipettor (Figures 4 & 5). If desired, commercially available magnetic stands from Ambion (Figure 6) and others can also be used.



Figures 4 & 5: Multiple-sample ChIP using ChIP-IT Express.
Immunoprecipitation and wash steps are fast and easy with magnetic beads because the pellet forms on the side of the tube, even pulling completely out of the buffer. Because it is simple to remove the buffer without disturbing the pellet, it is now possible to perform multiple ChIPs in PCR strips using a multi-channel pipettor.

Figure 6: Use of an Ambion 6 Tube Magnetic Stand with ChIP-IT Express.
Commercially available magnetic tube racks, such as those from Ambion and Promega, can be used with ChIP-IT Express.

Step 6: Reverse cross-links, chromatin elution & Proteinase K treatment

Following the wash steps, the chromatin is eluted from the antibody that is bound to the protein G beads. The specialized Elution and Reverse Cross-linking Buffers are optimized to reduce incubation times and also decrease background. The sample is then incubated with Proteinase K to digest the protein, followed by addition of Proteinase K Stop Buffer. The ChIP-IT Express protocol is unique because the buffers used for elution and digestion eliminate the need to purify the DNA before it is analyzed. With traditional ChIP, the DNA would have to be subjected to phenol/chloroform extractions, precipitation and resuspension. These steps are not necessary with ChIP-IT Express as the DNA is now ready for analysis. This saves time, minimizes manipulations and eliminates DNA loss that can occur during purification.

Step 7: PCR analysis using appropriate controls

The final step is to perform PCR using appropriate primers to determine if the expected DNA sequences were immunoprecipitated by your antibody. This confirms that the protein of interest was bound to the DNA when the cells were fixed (Figure 7).

Because ChIP is an enrichment of DNA bound by a particular protein, not a complete purification of the protein-bound DNA, ChIPs are unavoidably contaminated with non-specifically captured chromatin, which can make data interpretation difficult due to the false positive PCR products. To overcome this problem, Active Motif offers species-specific ChIP-IT Control Kits for human, mouse and rat, which provide one positive and one negative antibody, positive control primers, PCR buffer and a DNA loading dye that makes your PCR reactions ready for loading. In addition, we offer Ready-to-ChIP Chromatin that has been optimally sheared by sonication and validated in ChIP. For additional information on the Control Kits and DNA, please visit our website.

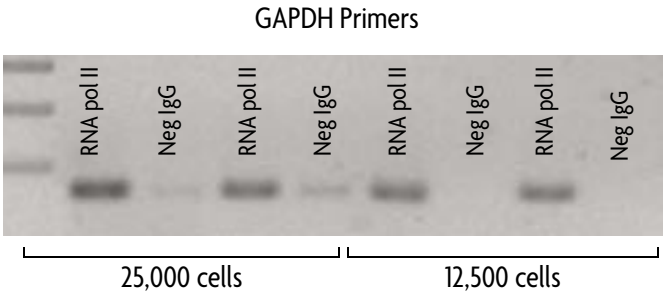


Figure 7: ChIP-IT Express works with 12,500 cells.
ChIP-IT Express was performed in duplicate on decreasing amounts of sonicated HeLa cell chromatin. Two µg of RNA pol II and Neg IgG antibody was used for IP. GAPDH PCR primers were used to analyze the immunoprecipitated DNA. Using the improved ChIP-IT Express reagents and protocol, positive ChIP data was obtained from as few as 12,500 cells. In addition, the simplified ChIP-IT Express protocol enabled ChIP to be performed in high-throughput using 8-tube PCR strips and a multi-channel pipettor together with the bar magnet included in the kit.

Conclusion

ChIP-IT Express Kits transform ChIP from a labor-intensive protocol that is appropriate for only a small number of samples into a rapid method that is suitable for experiments requiring multiple samples (*e.g.* studies of pathway induction, time-course experiments, *etc.*). The kits incorporate all of the improvements described above and include the reagents required to perform your choice of either enzymatic- or sonication-type chromatin shearing, and perform 25 ChIP reactions, including a powerful bar magnetic. For complete details, and to download the comprehensive protocol, please visit our website at www.activemotif.com/chip.